

# Inhibition of Mature IL-1 $\beta$ Production in Murine Macrophages and a Murine Model of Inflammation by WIN 67694, an Inhibitor of IL-1 $\beta$ Converting Enzyme<sup>1</sup>

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The proinflammatory cytokine IL-1 $\beta$  is synthesized by activated monocytes and macrophages as a 31-kDa, biologically inactive precursor that is proteolytically processed to the biologically active 17-kDa mature molecule by the IL-1 $\beta$  converting enzyme (ICE). WIN 67694, Z-Val-Ala-Asp-CH<sub>2</sub>O(CO)[2,6-(Cl<sub>2</sub>)Ph], is a potent, selective inhibitor of human ICE. In activated murine peritoneal macrophages, WIN 67694 inhibited the release of mature IL-1 $\beta$  with an IC<sub>50</sub> of 1.8  $\mu$ M without any effect on the release of IL-1 $\alpha$ , IL-6, or TNF- $\alpha$ . The effect was specific to mature IL-1 $\beta$  release; the ICE inhibitor did not effect IL-1 $\beta$  RNA levels or precursor protein synthesis. In vivo, WIN 67694 was also able to inhibit selectively the release of IL-1 $\beta$  in a dose-dependent manner in a subcutaneous tissue chamber implant model of inflammation. IL-1 $\beta$  levels in tissue chamber fluid were inhibited 35 and 55% at 10 and 100 mg/kg, respectively. IL-1 $\alpha$ , IL-6, and TNF- $\alpha$  levels were not affected. The ability to selectively inhibit mature IL-1 $\beta$  release in vivo with ICE inhibitors will allow for detailed studies of the role of IL-1 $\beta$  and ICE in inflammatory diseases. *The Journal of Immunology*, 1995, 154: 1331–1338.

**T**he proinflammatory cytokine IL-1 $\beta$  is thought to play a key role in several chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease (1, 2). IL-1 $\beta$  is synthesized in activated monocytes and macrophages as a 31-kDa biologically inactive precursor molecule that is proteolytically processed to produce a biologically active 17-kDa carboxy terminal polypeptide (3, 4). This cleavage is mediated by the IL-1 $\beta$  converting enzyme, or ICE<sup>3</sup>, a novel

cysteine protease (5, 6). The importance of ICE in the production of biologically active IL-1 $\beta$  is demonstrated by the ability of ICE inhibitors to inhibit the production of mature IL-1 $\beta$  from activated monocytes and macrophages (6, 7).

Both human and murine forms of ICE have recently been cloned and sequenced (6, 8–10). The two enzymes are highly conserved and exhibit absolute identity at the active site thiol and adjacent residues. The conservation between human and murine ICE suggests that murine models should be appropriate for studying the biologic and pharmacologic consequences of ICE inhibition. Indeed, we show in this study, as has been shown in other studies (11), that human ICE inhibitors retain a high degree of potency in the murine system.

To explore further the biologic and pharmacologic effects of ICE inhibitors, we have characterized the effect of a potent, selective, and irreversible inhibitor of ICE on the production of mature IL-1 $\beta$  from murine peritoneal macrophages in vitro and in inflammatory exudates in vivo. We demonstrate that selective inhibition of mature IL-1 $\beta$  generation is achievable in vivo, thereby creating new avenues into studies of the roles of ICE and mature IL-1 $\beta$  in animal models.

Received for publication July 11, 1994. Accepted for publication October 28, 1994.

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<sup>3</sup> Abbreviations used in this paper: ICE, IL-1 $\beta$  converting enzyme; FBS, fetal bovine serum; TBS, Tris-buffered saline (150 mM NaCl, 20 mM Tris, pH 7.5).

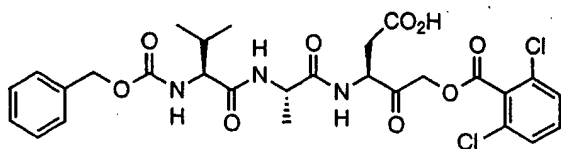


FIGURE 1. Structure of WIN 67694.

## Materials and Methods

### Materials

RPMI 1640 was obtained from Mediatech, Inc. (Herndon, VA). Heat-inactivated and dialyzed fetal bovine serum (FBS) were obtained from HyClone Laboratories (Logan, UT). Tran<sup>35</sup>S-label ([<sup>35</sup>S]methionine/[<sup>35</sup>S]-cysteine) and [<sup>32</sup>P]UTP were obtained from ICN Radiochemicals (Irvine, CA). ELISA kits for murine IL-1 $\alpha$  and TNF- $\alpha$  were purchased from Genzyme Corporation (Boston, MA). WIN 67694, Z-Val-Ala-Asp-CH<sub>2</sub>O(CO)[2,6-(Cl<sub>2</sub>)Ph] (Fig. 1), was synthesized as recently described (12). This compound has a second-order rate constant of 432,000 M<sup>-1</sup> s<sup>-1</sup> against human ICE and inhibits IL-1 $\beta$  secretion from human monocytes with an IC<sub>50</sub> of 0.5  $\mu$ M (7, 12).

### Peritoneal macrophage isolation and activation by LPS

Female, BALB/c mice 8 to 10 wk old (Taconic, Germantown, NY), were injected with 1 ml of 3% Brewer's thioglycolate, and peritoneal exudate cells were isolated 4 to 5 days later by peritoneal lavage with calcium/magnesium-free PBS containing 0.1% glucose. Cells were washed and resuspended in RPMI 1640/25 mM HEPES/5% heat-inactivated FBS at  $1.25 \times 10^6$  cells/ml. Macrophages were purified by plating  $2.5 \times 10^6$  cells/well in 6-well plates (Corning, Corning, NY). Cells were allowed to adhere for 2 h, then nonadherent cells were removed by washing. Adherent cells were stimulated with 1  $\mu$ g/ml LPS (*Salmonella minnesota* Re595, Calbiochem, La Jolla, CA) for 2 h. To stimulate the release of mature IL-1 $\beta$ , cells were washed and subsequently incubated for 30 min with 40  $\mu$ M nigericin (Sigma Chemical Co., St. Louis, MO) (13). After incubation with nigericin, culture media was collected and centrifuged at  $4000 \times g$  for 10 min. The supernatants were then analyzed for cytokine levels by ELISA.

### Pulse-labeling assay for IL-1 $\beta$ synthesis and secretion

Adherent macrophages were isolated as described above and stimulated with 1  $\mu$ g/ml LPS for 90 min. The cells were then washed and incubated for 15 min with cysteine/methionine-free RPMI 1640 containing 1% dialyzed FBS and 1  $\mu$ g/ml LPS, and then 100  $\mu$ Ci/ml of Tran<sup>35</sup>S-label was added. The cells were then incubated for an additional 60 min. For determination of IL-1 $\beta$  synthesis, the media was removed immediately after the labeling step, and cells were lysed by addition of RIPA buffer containing protease inhibitors (14). For measurement of secretion of mature IL-1 $\beta$ , the labeled cells were incubated with 40  $\mu$ M nigericin for 30 min before the collection of media. Lysates and media samples were cleared by centrifugation at  $12,000 \times g$  for 10 min. An aliquot of the lysate was immunoprecipitated with goat anti-murine IL-1 $\beta$  IgG (R&D Systems, Minneapolis, MN). Put briefly, samples were preabsorbed by rotating for 30 min at 4°C with 50  $\mu$ l of a 50% slurry of protein G-Sepharose, centrifuged at  $12,000 \times g$ , and the supernatants transferred to new tubes. After the addition of goat anti-murine IL-1 $\beta$  IgG (15  $\mu$ g/ml) (R&D Systems), samples were rotated overnight at 4°C. Fifty microliters of protein G-Sepharose was then added, and samples were rotated for 1 h at 4°C. The immunoprecipitates were washed three times with RIPA buffer and resuspended in SDS-PAGE sample buffer containing 6% mercaptoethanol (15). After heating at 95°C for 3 min, samples were fractionated by electrophoresis on 15% SDS polyacrylamide gels. Gels were treated with Amplify (Amersham, Arlington Heights, IL), dried, and placed on film (Kodak XAR-5) overnight. Bands corresponding to IL-1 $\beta$  were quantitated by laser densitometry.

### mRNA isolation and Northern analysis

Thioglycolate-elicited peritoneal macrophages ( $1 \times 10^7$ ) were incubated in RPMI 1640/5% FBS/25 mM HEPES with 1  $\mu$ g/ml LPS for 3.5 h.

Total cellular RNA was prepared as described (16). Five micrograms each RNA sample was size-fractionated on a 1.0% agarose/2.2 M formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham) according to standard protocols (17). Blots were probed with murine IL-1 $\beta$  and rat cyclophilin cRNAs as described previously (18). Put briefly, blots were prehybridized for 2 h at 57°C in a 50% formamide, 50  $\mu$ g/ml heparin buffer, hybridized for 16 h at 57°C in the same buffer, and washed twice at 65°C in 0.2X SSC/0.2% SDS. Autoradiography was conducted at -70°C using Kodak XAR film and Fisher Lightening Plus intensifying screens. Bands were quantitated by laser densitometry. The <sup>32</sup>P-labeled murine IL-1 $\beta$  cRNA probe was synthesized by in vitro transcription of a full-length murine IL-1 $\beta$  cDNA in pSG-BSmIL-1 $\beta$  (+) (kind gift of Dr. David Chaplin: 19). The <sup>32</sup>P-labeled cyclophilin cRNA probe was synthesized by in vitro transcription of a cDNA representing nucleotides 42 to 520 of the rat cyclophilin mRNA (20). The cyclophilin cDNA was generated by reverse transcriptase-PCR using rat whole knee joint RNA as a template and was ligated into pCRII (Invitrogen, San Diego, CA) for in vitro transcription.

### Synthesis of <sup>35</sup>S-labeled precursor IL-1 $\beta$

<sup>35</sup>S-labeled murine proIL-1 $\beta$  was generated by in vitro transcription and translation of pSG5 mIL-1 $\beta$  using a TnT kit (Promega, Madison, WI). pSG5 mIL-1 $\beta$  contains a full-length murine proIL-1 $\beta$  cDNA (19) and was constructed by inserting the 1.4-kb IL-1 $\beta$  EcoRI cDNA fragment of pSG-BSmIL-1 $\beta$  (+) into pSG5 (Stratagene, La Jolla, CA).

### ICE activity in cell lysates

Macrophage lysates were prepared by four freeze-thaw cycles. The lysates were centrifuged at  $16,000 \times g$  for 20 min, and ICE activity was measured in the supernatants. An 18- $\mu$ l aliquot of the cell lysate added to tubes containing 1  $\mu$ l of [<sup>35</sup>S]methionine-labeled murine precursor IL-1 $\beta$  and 11  $\mu$ l of assay buffer (30% glycerol, 30 mM Tris, pH 8.0). Tubes were incubated for 60 min at 30°C. The reactions were stopped by the addition of 15  $\mu$ l of 4X SDS sample buffer containing 25% 2-ME. Aliquots were fractionated on 15% SDS polyacrylamide gels as described above.

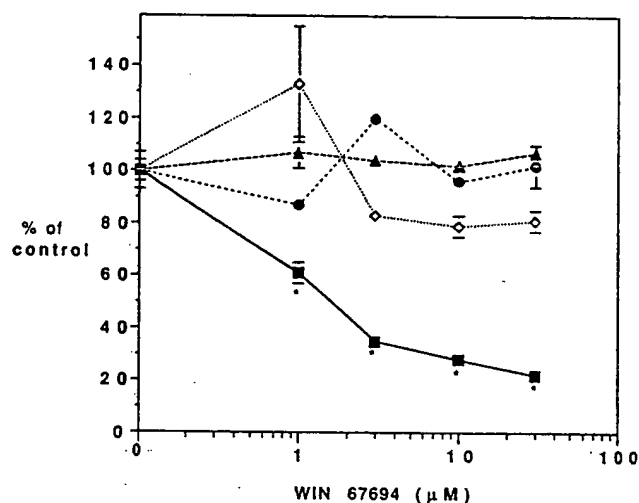
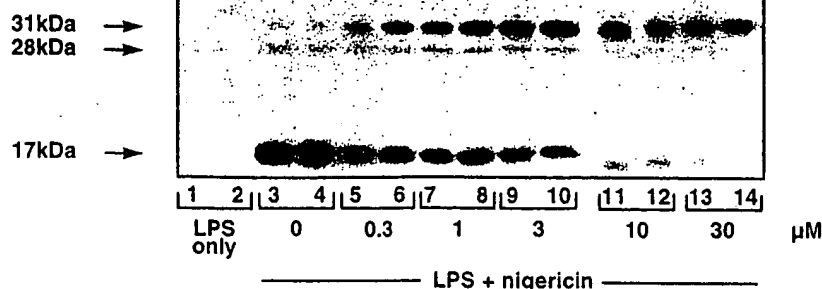
### Murine IL-1 $\beta$ ELISA

ELISA plates (Costar high binding plates) were coated with a hamster mAb to murine IL-1 $\beta$  (Genzyme) (5  $\mu$ g/ml) in PBS overnight at 4°C, washed, and blocked with 3% BSA in Tris-buffered saline (TBS) for 1 h. Aliquots (100  $\mu$ l) of diluted samples were added and incubated for 2 h at room temperature. The plates were washed five times and incubated for 1 h at room temperature with 10  $\mu$ g/ml of goat anti-murine IL-1 $\beta$  IgG (R&D Systems). After five washes, the plates were incubated for 1 h at room temperature with a 1:2000 dilution of an alkaline phosphatase-conjugated mouse anti-goat IgG. After four washes, the plates were incubated with substrate solution (1 mg/ml p-nitrophenylphosphate in alkaline buffer, Sigma Chemical Co.). Color development was quantitated by reading the OD<sub>405</sub> on a plate spectrophotometer (Molecular Devices Corporation, Menlo Park, CA).

### Specificity of murine IL-1 $\beta$ ELISA

To determine which forms of IL-1 $\beta$  were detected by the ELISA assay, a sample containing <sup>35</sup>S-labeled precursor and mature IL-1 $\beta$  derived by combining samples of secreted material (mature IL-1 $\beta$ ) and cell lysates (precursor IL-1 $\beta$ ) was incubated in wells coated with the hamster mAb to murine IL-1 $\beta$ . After a 1 h incubation, unbound material was removed by washing, and the bound material was solubilized by adding 100  $\mu$ l of SDS-sample buffer and incubating for 30 min at room temperature. Bound material was fractionated by SDS-PAGE followed by fluorography as described above. Under the conditions of the ELISA assay, only mature IL-1 $\beta$  was bound to the plates, and no binding of precursor IL-1 $\beta$  was observed (data not shown). The ELISA was able to detect mature IL-1 $\beta$  over the range 10 to 10,000 pg/ml and did not detect murine IL-1 $\alpha$ , IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , or IFN- $\gamma$  at concentrations up to 100 ng/ml.

**FIGURE 2.** Pulse-chase analysis of IL-1 $\beta$  secretion from murine peritoneal macrophages. Adherent macrophages were stimulated with 1  $\mu$ g/ml LPS and labeled with Tran<sup>35</sup>S-label for 60 min followed by incubation with 40  $\mu$ M nigericin for 30 min. IL-1 $\beta$  was immunoprecipitated from the media and analyzed by SDS-PAGE. WIN 67694 was added to the cells after the adherence step and was present for the remainder of the experiment.



**FIGURE 3.** Effect of WIN 67694 on cytokine release by LPS-stimulated peritoneal macrophages. Adherent macrophages were stimulated with 1  $\mu$ g/ml LPS for 2.5 h followed by 40  $\mu$ M nigericin for 30 min. Cytokine levels were measured by ELISA in the culture media. ■, IL-1 $\beta$ ; ◇, IL-1 $\alpha$ ; ●, IL-6; ▲, TNF- $\alpha$ . Results are means  $\pm$  SEM. \* $p$  < 0.05 compared with control value.

#### Murine IL-6 ELISA

Procedures were identical to that described above for the IL-1 $\beta$  ELISA except for the following: 1) plates were coated with a rat mAb to murine IL-6 (Upstate Biotechnology, Inc., Lake Placid, NY) (1  $\mu$ g/ml in PBS) and 2) after the sample incubation step, plates were incubated with 2  $\mu$ g/ml of goat anti-murine IL-6.

#### Cytokine production in vivo

To study IL-1 $\beta$  production in vivo, we employed a model recently described by Dawson et al. (21). Cylindrical Teflon chambers, 20 mm  $\times$  10 mm, (kindly provided by Dr. Klaus Vosbeck, Ciba-Geigy, Basle) were implanted subcutaneously into the backs of female BALB/c mice under isoflurane anesthesia. After chamber implantation, the mice were allowed to recover for 14 days. An inflammatory response was initiated in the chamber by the injection of a suspension of 1% zymosan A (Sigma Chemical Co.) in pyrogen-free saline (Baxter, Deerfield, IL) through one of the chamber's access holes. Six h after zymosan administration, an aliquot of the chamber fluid was collected by inserting a 20-gauge needle into the chamber and aspirating the fluid. The fluid was centrifuged at 1000  $\times$  g for 10 min to remove free cells, and the supernatant was stored at  $-20^{\circ}\text{C}$  for ELISA analysis. For experiments in which the inhibition of IL-1 $\beta$  production by WIN 67694 was examined, the compound was prepared in polyethylene glycol-400/water (80%/20%, v/v) at 10 or 20

mg/ml and administered by intraperitoneal injection at 1, 3, and 5 h after zymosan injection.

#### WIN 67694 quantitation in plasma and tissue chamber fluid

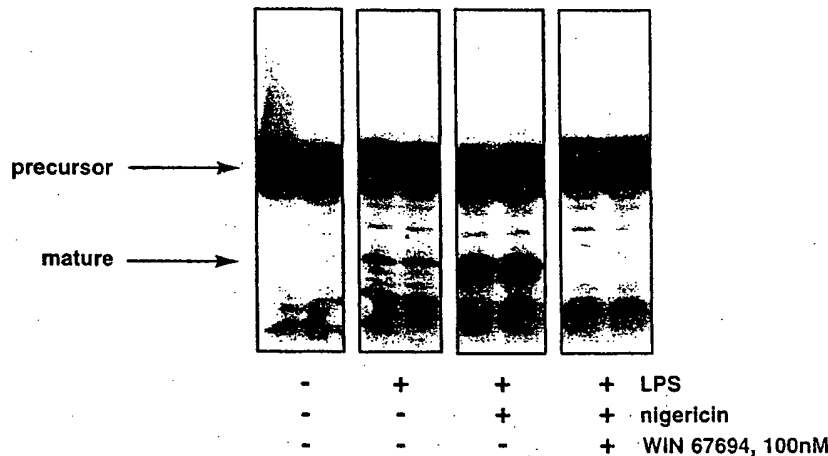
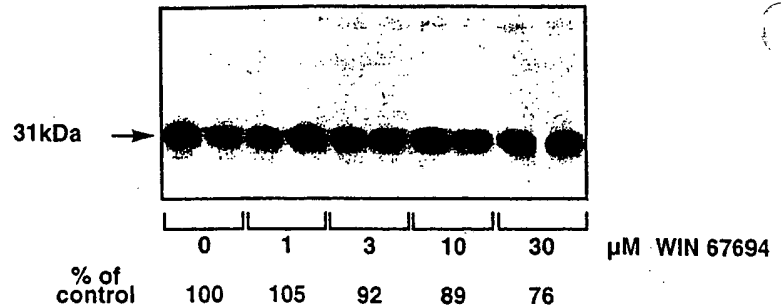
WIN 67694 was quantified in the plasma and tissue chamber fluid samples by an in vitro fluorogenic ICE bioassay. The samples were initially diluted in assay buffer (0.01 M HEPES (pH 7.5), 25% glycerol) to solutions containing 6% plasma or tissue chamber fluid. Subsequent dilutions were prepared using 6% normal plasma or tissue chamber fluid in assay buffer. A standard curve of WIN 67694 was prepared in assay buffer containing either 6% normal plasma or tissue chamber fluid. The samples and standards were preincubated with human recombinant ICE for 15 min at  $37^{\circ}\text{C}$ , and the enzyme reactions were initiated with substrate, Suc-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin (Bachem). The final reaction mixtures, which contained 0.01 M HEPES (pH 7.5), 25% glycerol, 1 mM DTT, 2% plasma or tissue chamber fluid, and 15  $\mu$ M ( $1 \times K_m$ ) substrate, were incubated for 30 min at  $37^{\circ}\text{C}$ . The reactions were terminated using 0.1N HCl and were neutralized with an equal volume of 1 M Tris (pH 10.5). Fluorescence was read at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan II plate reading fluorimeter (Labsystems, Marlboro, MA). The data were fit using the four-parameter  $\text{IC}_{50}$  equation, and the results from the standard curve were used to calculate concentrations for unknowns that fell between 20 and 80% of control. Known concentrations of WIN 67694 also were run in the bioassay, and the concentrations calculated from the standard curve fell within 20% of the expected values.

## Results

#### IL-1 $\beta$ secretion from peritoneal macrophages

Immunoprecipitation of culture media from LPS-stimulated macrophages labeled with Tran<sup>35</sup>S-label indicated that cells stimulated with only LPS released no detectable IL-1 $\beta$  (Fig. 2, lanes 1 and 2). This result is similar to that recently reported by other investigators (13, 22, 23). In contrast, treatment of LPS-stimulated macrophages with nigericin resulted in secretion of a large amount of predominantly mature IL-1 $\beta$  (Fig. 2, lanes 3 and 4). Mature IL-1 $\beta$  accounted for greater than 95% of the total IL-1 $\beta$  detected extracellularly. The induction of mature IL-1 $\beta$  release in response to nigericin is similar to that reported by Perregaux et al. (13). The effect of this  $\text{K}^+/\text{H}^+$  ionophore (24) cannot be explained simply by cell lysis because it has been shown that nonspecific lytic agents cause release of large amounts of only precursor IL-1 $\beta$  (13, 23), and high extracellular levels of  $\text{K}^+$  present during nigericin treatment can block both the nigericin-induced release of mature IL-1 $\beta$  and the nigericin-induced increase in ICE

**FIGURE 4.** Synthesis of precursor IL-1 $\beta$  by murine peritoneal macrophages. Adherent peritoneal macrophages were stimulated with 1  $\mu$ g/ml LPS for 90 min and labeled with Tran-<sup>35</sup>S-label for 60 min. Cells were lysed by addition of RIPA buffer containing protease inhibitors. Precursor IL-1 $\beta$  was isolated from cell lysates by immunoprecipitation and analyzed by SDS-PAGE and laser densitometry.



**FIGURE 5.** ICE activity in murine peritoneal macrophage cell lysates. Cell lysates were prepared and ICE activity was measured as described in *Materials and Methods*.

activity in cell lysates (B. E. Miller, D. M. Gauvin, K. B. Holbrook, manuscript in preparation). A small amount of full-length precursor IL-1 $\beta$  was also detected in the extracellular media of nigericin-treated cells as was a minor amount of a 28-kDa processed IL-1 $\beta$  product that is produced as a result of cleavage by ICE at the Asp<sup>27</sup>-Gly<sup>28</sup> bond, a secondary cleavage site (3, 5, 25). Release of mature IL-1 $\beta$  was inhibited in a dose-dependent manner by the ICE inhibitor WIN 67694 with an IC<sub>50</sub> of 1.8  $\mu$ M (Fig. 2). The inhibition of mature IL-1 $\beta$  release was associated with increased release of precursor IL-1 $\beta$ . This result is similar to the that in the studies with human monocytes reported by Thornberry et al. (6) and Uhl et al. (7). ELISA measurements confirmed the inhibition of mature IL-1 $\beta$  release seen with the pulse-chase assay and showed that WIN 67694 treatment did not affect the release of IL-1 $\alpha$ , IL-6, and TNF- $\alpha$ , demonstrating the specificity of WIN 67694 action on the production of mature IL-1 $\beta$  (Fig. 3).

#### IL-1 $\beta$ mRNA expression and precursor synthesis

To determine if WIN 67694 affected the induction of IL-1 $\beta$  mRNA expression during LPS activation, macrophages were incubated with LPS in the presence or absence of 30  $\mu$ M WIN 67694. Treatment with WIN 67694 during the activation period had no effect on the level of IL-1 $\beta$  mRNA as measured by Northern analysis (data not shown). To determine if WIN 67694 affected precursor IL-1 $\beta$  synthesis, LPS-stimulated macrophages were pulse-

labeled with Tran<sup>35</sup>S-label, and synthesis of IL-1 $\beta$  was measured by quantitating the amount of immunoprecipitated precursor IL-1 $\beta$  in the cell lysates. WIN 67694 at concentrations up to 10  $\mu$ M had no effect on IL-1 $\beta$  synthesis (Fig. 4). At 30  $\mu$ M, a 24% reduction in precursor IL-1 $\beta$  synthesis was observed.

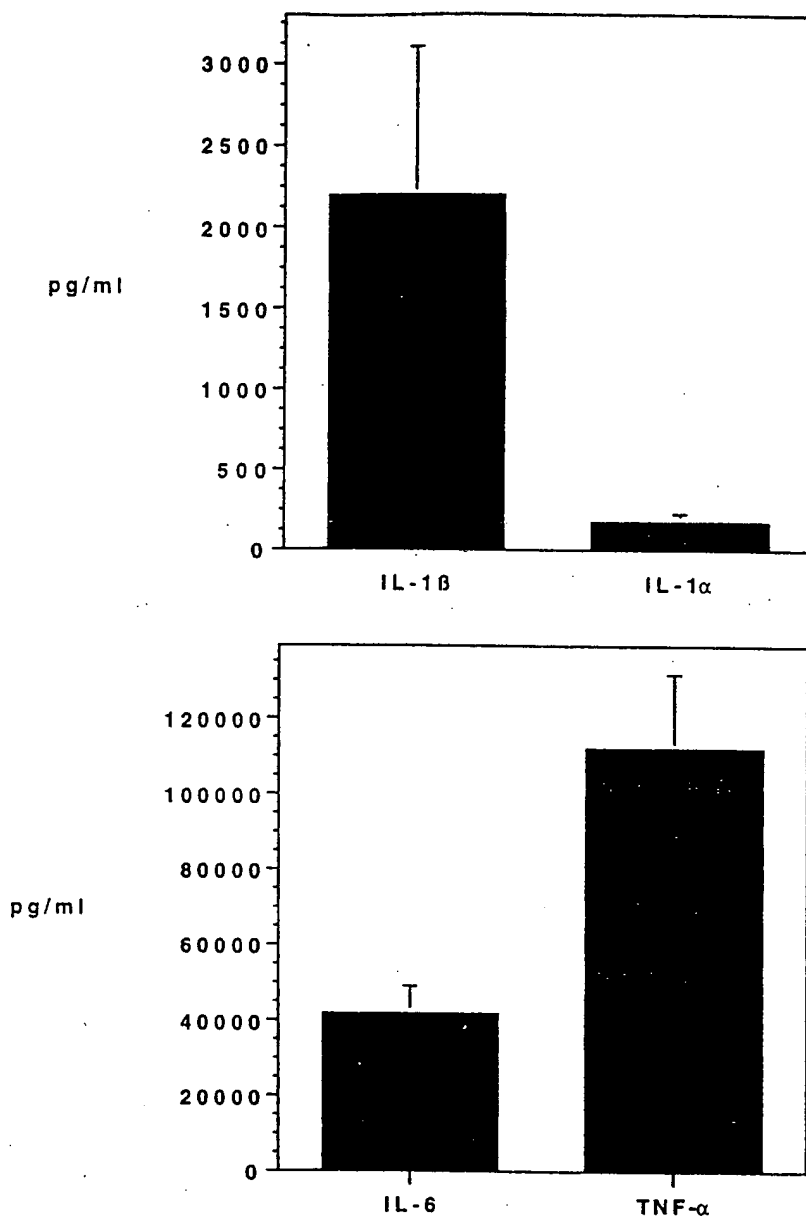
#### IL-1 $\beta$ converting enzyme activity

ICE activity in cell lysates was determined by assessing the conversion of exogenous, radiolabeled precursor IL-1 $\beta$  to mature IL-1 $\beta$ . Unstimulated macrophages contained a low level of enzyme activity (Fig. 5). However, stimulation of cells with LPS alone for 2.5 h resulted in increased enzyme activity. Treatment with nigericin after LPS stimulation (the same regimen used to induce the release of mature IL-1 $\beta$ ) resulted in a large increase in ICE activity compared with that in unstimulated cells or cells treated with LPS alone. The ICE activity in macrophage lysates was inhibited by WIN 67694.

#### IL-1 $\beta$ secretion in vivo

As reported recently by Dawson et al. (21), the subcutaneous implantation of small, plastic chambers into mice provides a readily accessible compartment for assessing cytokine secretion in vivo. Injection of zymosan into the chambers was reported to cause the production of large amounts of IL-1 $\beta$  and IL-6 (21). In our study, we saw

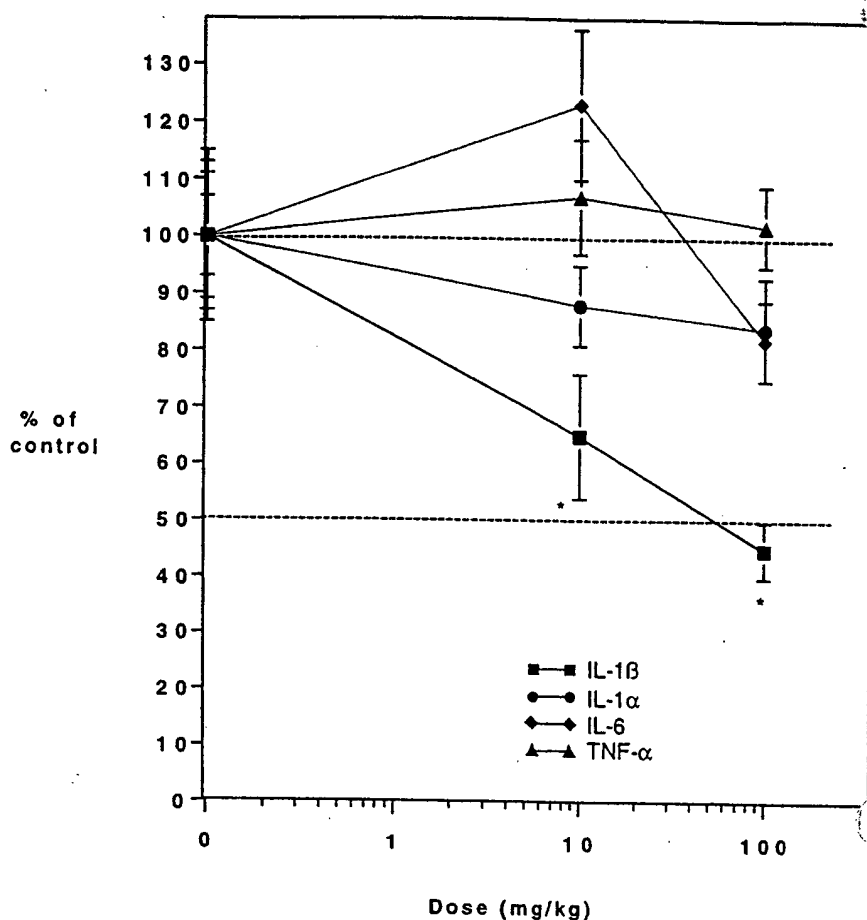
FIGURE 6. Cytokine levels in tissue chamber fluid. Subcutaneously implanted tissue chambers were injected with 500  $\mu$ l of a 1% suspension of zymosan A in saline and tissue chamber fluid collected 6 h later. Cytokine levels were measured by specific ELISAs. Values are means  $\pm$  SEM.



similar results along with high levels of both IL-1 $\alpha$  and TNF- $\alpha$  (Fig. 6). Thus, this model may be useful for looking at the effects of anti-inflammatory agents on the production of a number of cytokines in vivo. To determine if IL-1 $\beta$  release could be inhibited in vivo, tissue chambers were injected with zymosan, followed by i.p. administration of WIN 67694 at 1, 3, and 5 h after zymosan administration. Preliminary experiments had determined that, because of rapid clearance of this compound in the mouse, this dosing regimen was necessary to maintain efficacious concentrations in plasma and tissue chamber fluid (B. E. Miller, D. M. Gauvin, K. G. Holbrook, unpublished observations). The half-life of WIN 67694 in plasma and tissue chamber fluid was 40

min and 150 min, respectively. WIN 67694 treatment resulted in a dose-dependent reduction in IL-1 $\beta$  levels in tissue chamber fluid (Fig. 7). IL-1 $\beta$  levels were reduced 35 and 55% below control levels at 10 mg/kg and 100 mg/kg, respectively. Levels of IL-1 $\alpha$ , IL-6, and TNF- $\alpha$  were not significantly affected by treatment with WIN 67694, thus demonstrating that an ICE inhibitor is able to selectively block the release of IL-1 $\beta$  in vivo. The concentration of WIN 67694 in plasma and tissue chamber fluid is shown in (Table 1). The percent reduction in IL-1 $\beta$  levels observed after in vivo treatment with WIN 67694 (35 and 55% inhibition at tissue chamber levels of 0.6 and 8.2  $\mu$ M, respectively) is consistent with what would be predicted based on the in vitro dose

**FIGURE 7.** Effect of WIN 67694 on cytokine levels in tissue chamber fluid. Subcutaneously implanted tissue chambers were injected with 500  $\mu$ l of a 1% suspension of zymosan A in saline. WIN 67694 was prepared as a solution in PEG-400/water and administered by i.p. injection 1, 3, and 5 h after zymosan. Tissue chamber fluid was collected 6 h after zymosan. Cytokine levels were measured by specific ELISA. ■, IL-1 $\beta$ ; ●, IL-1 $\alpha$ ; ◆, IL-6; ▲, TNF- $\alpha$ . Values are mean  $\pm$  SEM. \* $p$  < 0.05 compared with control. The dashed lines indicate 100 and 50% of the control values. The experiment was repeated two times with similar results.



**Table I.** WIN 67694 concentrations in plasma and tissue chamber fluid after i.p. administration

Dose (mg/kg)	Concentration (ng/ml)*	
	Plasma	Tissue chamber fluid
10	1,118 $\pm$ 164 (1.8)	348 $\pm$ 72 (0.6)
100	90,799 $\pm$ 4,952 (146)	5,117 $\pm$ 1,058 (8.2)

\* Values are means  $\pm$  SEM. Concentration in  $\mu$ M is shown in parentheses.

response for inhibition of IL-1 $\beta$  secretion from peritoneal macrophages in which inhibition at 1  $\mu$ M was approximately 35% and inhibition at 10  $\mu$ M was approximately 75%.

## Discussion

Several studies have demonstrated that peptide-based inhibitors of human ICE can selectively block release of IL-1 $\beta$  from human peripheral blood monocytes without effect on the release of other cytokines. In this study, we have shown that an ICE inhibitor is able to selectively

block the release of IL-1 $\beta$  from activated murine macrophages. More importantly, we have shown that mature IL-1 $\beta$  production in vivo can be inhibited by parenteral administration of an ICE inhibitor. To our knowledge, this is the first demonstration that an ICE inhibitor can selectively inhibit mature IL-1 $\beta$  production in vivo.

Although the precise role of IL-1 $\beta$  in disease pathogenesis is still unclear, several lines of evidence implicate IL-1 $\beta$  as a contributory or causative factor in the pathogenesis of rheumatoid arthritis and several other inflammatory diseases. IL-1 $\beta$  levels have been shown to correlate with disease activity in synovial fluid from rheumatoid arthritis patients (26–28). Several recent studies (29, 30) with animal models of arthritis have shown that Abs to IL-1 reduce both clinical indicators of disease and the histologic evidence of joint damage. Although a more pronounced effect was seen with Abs to both IL-1 $\alpha$  and IL-1 $\beta$ , Abs to IL-1 $\alpha$  alone were ineffective whereas Abs to IL-1 $\beta$  resulted in a significant reduction in disease parameters. In addition, inhibition of IL-1 activity with the IL-1 receptor antagonist has been shown to block several events important in arthritic disease, including IL-1-induced metalloproteinase induction in chondrocytes and synovial lining cells and cartilage matrix degradation in explant cultures (31–33).

No studies on the function of ICE in vivo or its role in inflammatory diseases have yet been reported. Precursor IL-1 $\beta$  can be released as a consequence of cell lysis or damage and can be processed to biologically active peptides in vitro by extracellular enzymes (e.g., elastase, cathepsin G, and stromelysin) commonly found at sites of an inflammatory response (34). The extent to which this actually occurs in vivo is unknown. However, the importance of ICE in generating an inflammatory response is strongly suggested by the recent results of Ray et al. (35). These investigators showed that the cowpox virus encodes for a 38-kDa ICE inhibitor. Lesions produced in response to infection by wild-type virus contained few inflammatory cells, whereas lesions produced by a mutant virus that lacked the 38-kDa ICE inhibitor were characterized by a large influx of inflammatory cells. These observations provide evidence supportive of a role for ICE in the generation of an inflammatory response in vivo. The potential advantages and disadvantages of ICE as a therapeutic target have been discussed in a recent review (36).

In summary, we have demonstrated that an ICE inhibitor is able to selectively inhibit the production of mature IL-1 $\beta$  both in vitro and in vivo. The development of potent and selective inhibitors of ICE that, as reported in this study, are active in vivo will permit detailed studies of the role of this enzyme and IL-1 $\beta$  in inflammatory disease.

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